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ATP-driven Na^+ transport and Na^+-dependent ATP synthesis in *Escherichia coli* grown at low $\Delta\bar{\mu}_{H^+}$

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In inverted subcellular vesicles of *Escherichia coli* grown at high $\Delta\bar{\mu}_{H^+}$ (neutral pH, no protonophorous uncoupler), ATP-driven Na^+ transport and oxidative phosphorylation are completely inhibited by the protonophore CCCP. If *E. coli* was grown at low $\Delta\bar{\mu}_{H^+}$, i.e. at high pH or in the presence of uncoupler, some oxidative phosphorylation was observed in the vesicles even in CCCP-containing medium, and Na^+ transport was actually stimulated by CCCP. The CCCP-resistant transport and phosphorylation were absent from the unc mutant lacking F_0F_1 ATPase. Both processes proved to be sensitive to (i) the Na^+/H^+ antiporter monensin, (ii) the Na^+ uniporter ETH 157, (iii) the F_0 inhibitors DCCD and venturicidin, and (iv) the F_1 inhibitor aurovertin. The CCCP-resistant oxidative phosphorylation was stimulated by Na^+ and arrested by oppositely directed ΔpNa. These data are consistent with the assumption that, under appropriate growth conditions, the F_0F_1-type ATPase of *E. coli* becomes competent in transporting Na^+ ions.

Low $\Delta\bar{\mu}_{H^+}$; Na^+-ATPase; Na^+-coupled oxidative phosphorylation; *Escherichia coli*

1. INTRODUCTION

Our group recently found that an Na^+-motive respiratory chain is induced in *E. coli* grown under low $\Delta\bar{\mu}_{H^+}$ conditions, i.e. in the presence of protonophore or at high pH [1–4]. Within the framework of the Na^+ cycle concept [4–6], it seemed reasonable to assume that the Na^+-motive respiratory chain is coupled to the Na^+-driven ATP-synthase to form ATP at the expense of the respiration-produced $\Delta\bar{\mu}_{Na^+}$. Sodium ion-coupled non-oxidative phosphorylation is already known to occur in anaerobic *Propionigenium modestum* where an Na^+-motive decarboxylase is used as the $\Delta\bar{\mu}_{Na^+}$ generator [7,8]. In our laboratory, Na^+-coupled oxidative phosphorylation [9,10] and ATP-driven Na^+ transport [11] have been shown in subcellular vesicles of *Vibrio alginolyticus*. Sodium ion-pumping ATPases were described in plasma membrane of animal cells, in *Streptococcus faecalis*, methanogenic bacteria, *Mycoplasma*, *Acholeplasma* (reviewed in [4]) and quite recently in an acetogenic bacterium [12] and in plasma membrane of a marine eucaryotic alga [13,14].

In this paper we describe ATP-dependent Na^+ transport and $\Delta\bar{\mu}_{Na^+}$-driven oxidative phosphorylation in inside-out subcellular vesicles of *E. coli* grown in the presence of the protonophore PCP or in an alkaline medium.

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Abbreviations: $\Delta\bar{\mu}_{H^+}$ and $\Delta\bar{\mu}_{Na^+}$, transmembrane differences in electrochemical H^+ and Na^+ potentials, respectively; $\Delta\Psi$, transmembrane electric potential difference; ΔpH and ΔpNa, transmembrane H^+ and Na^+ concentration gradients; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexyl cabrodiimide; ETH 157, *N,N'*-dibenzyl-*N,N'*-diphenyl-1,2-phenylene diacetamide; PCP, pentachlorophenol.

2. MATERIALS AND METHODS

The *E. coli* strains K-12 GR 70N (F^-, gal rps^L str^R thi^-) and K-12 LE392 Δunc B-D [15,16] were generous gifts from Prof. R.B. Gennis and Prof. W.S.A. Brusilow, respectively. In the majority of experiments, the growth medium contained 10 mM NaCl, 20 mM sodium phosphate, 22 mM potassium phosphate, 1 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, thiamine (1 μg/ml), streptomycin (0.1 mg/ml), 50 mM sodium succinate, 50 mM glycyl glycine, pH 8.6 or 7.2, and 0.05% yeast extract. When indicated, 1×10^{-4} M PCP and/or 0.4% glycerol (v/v) were added. The cells were grown at 37°C for 12 h. The final absorbance at 600 nm was 0.7–1.2 O.D.

To prepare inverted subcellular vesicles, the cells were harvested by centrifugation (7,500 × *g*, 10 min, 4°C). The sediment was washed twice with 150 mM NaCl, 5 mM $MgSO_4$ and 10 mM Tris-sulfate, pH 8.2. The washed cells were suspended in solutions of the following composition: (1) for the Na^+ transport measurements, 50 mM K_2SO_4, 5 mM Na_2SO_4, 5 mM $MgSO_4$, 0.5 mM EDTA, 4 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, bovine serum albumin (1 mg/ml), DNase I (0.01 mg/ml), 100 mM Tricine-KOH, pH 7.5, or (2) for the ATP synthesis measurements, 250 mM Na_2SO_4, 30 mM $MgSO_4$, 0.5 mM EDTA, 4 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, bovine serum albumin (1 mg/ml), DNase I (0.01 mg/ml), 100 mM bis-Tris propane sulfate, pH 9.5. The suspension was treated with

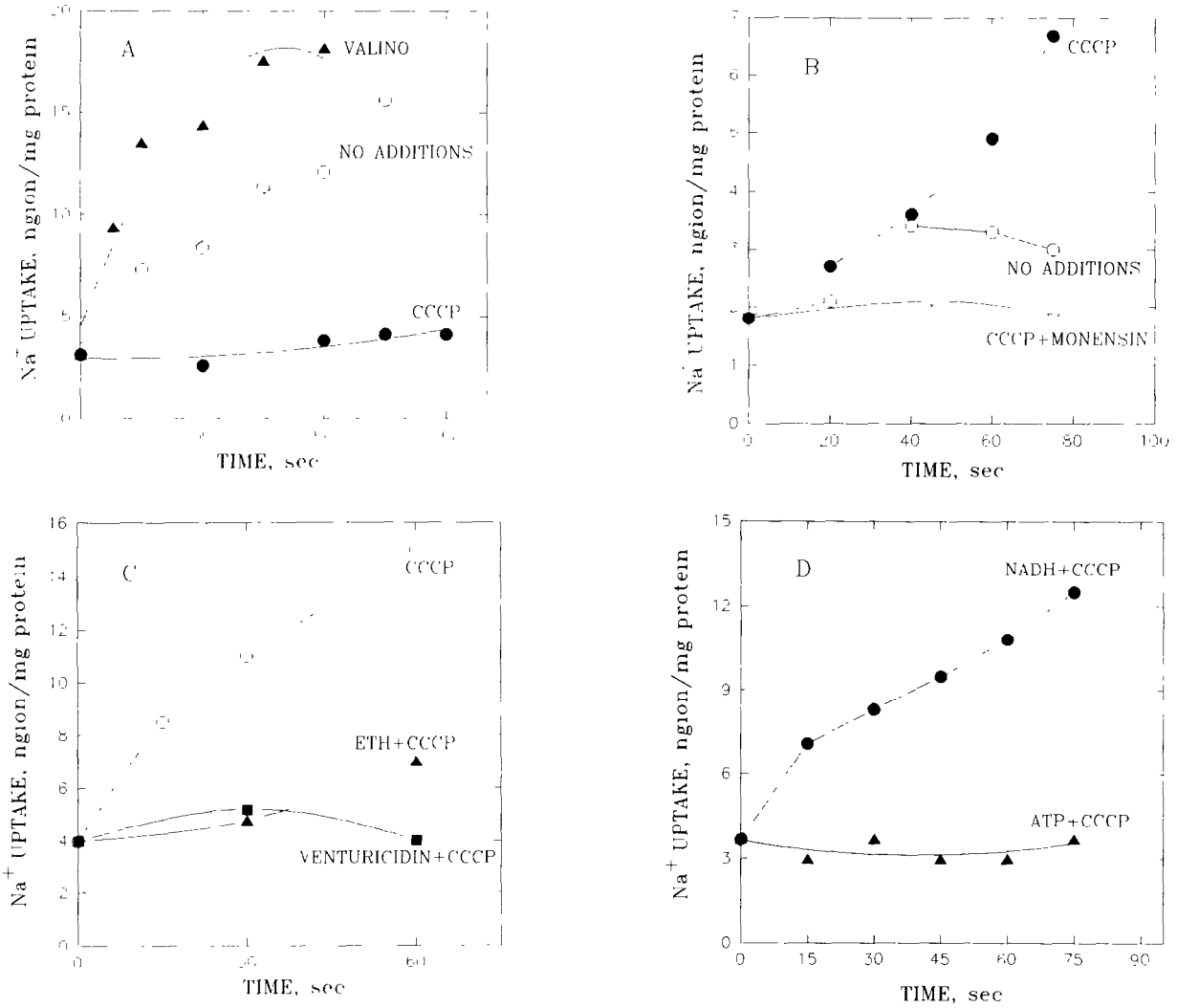

Fig. 1. The ATPase-driven Na^+ uptake by inverted subcellular vesicles of *E. coli* K-12 GR 70N (A–C) or K-12 LE 392 Δunc (D) grown at pH 7.2 (A), at pH 7.2 with 1×10^{-4} M PCP (B), at pH 8.6 (C) or at pH 8.6 with 1×10^{-4} M PCP (D). At zero time, 5 mM ATP-Tris was added. Where indicated, the incubation mixture (see section 2) was supplemented with 1×10^{-5} M valinomycin, 1×10^{-5} M CCCP, 2×10^{-5} M monensin, venturicidin (16 μg/mg protein) or 1×10^{-4} M ETH 157.

Aminco French press (1,000 psi) and then incubated for 10 min at 25°C. The cell debris was removed by centrifugation at $14,000 \times g$ for 15 min, at 2°C. The vesicles were sedimented by centrifugation of the supernatant at $50,000 \times g$ for 90 min, at 2°C. The sediment was suspended in solutions (1) or (2) to obtain the protein concentrations of 45–50 or 180–200 mg/ml, respectively.

The following incubation mixture was used in the Na^+ transport experiments: 50 mM K_2SO_4, 30 mM $MgSO_4$, 10 mM NaF, 10 mM tricyclohexyl ammonium phosphoenolpyruvate, 0.2 mM EDTA, 100 mM Tricine-KOH, pH 7.75, pyruvate kinase (20 units/ml). The reaction was started by adding ATP. Before the addition of ATP, the vesicles were pre-incubated with DCCD (4–5 nmol/mg protein) for 5 min. Both pre-incubation and incubation were carried out at room temperature. The Na^+ uptake by vesicles was measured as described elsewhere [2,3].

In the ATP synthesis experiments, the vesicles (10–20 mg protein/ml) were pre-incubated for 5 min at 30°C with DCCD (3–6 nmol/mg protein) in a mixture of 0.8 M sucrose, 30 mM $MgSO_4$, 0.2 mM EDTA, 5 mM bis-Tris-propane sulfate, pH 9.5, 1 mM ADP. Then the mixture was diluted ten-fold with a solution of 0.7 M sucrose, 2 mM potassium phosphate, 30 mM $MgSO_4$, 0.2 mM EDTA, 100 mM bis-Tris-propane sulfate, pH 6.5, and ATP Monitoring Reagent (LKB). The ATP formation was measured with the Luminometer 1251 (LKB).

3. RESULTS AND DISCUSSION

Fig. 1A shows the ATP-supported Na^+ uptake by the inverted subcellular vesicles of *E. coli* grown at pH 7.2 without uncoupler. One can see that the Na^+ transport was stimulated by valinomycin and completely abolished by protonophorous uncoupler CCCP. These relationships can be easily explained assuming that H^+-ATPase produces $\Delta\Psi$ which is converted to ΔpH by the valinomycin-mediated K^+ efflux. The ΔpH formed is utilized by the Na^+/H^+ antiporter to take up Na^+. Both $\Delta\Psi$ and ΔpH are discharged by CCCP so that ATP hydrolysis cannot support the Na^+ transport.

A quite different inhibitor pattern was revealed when

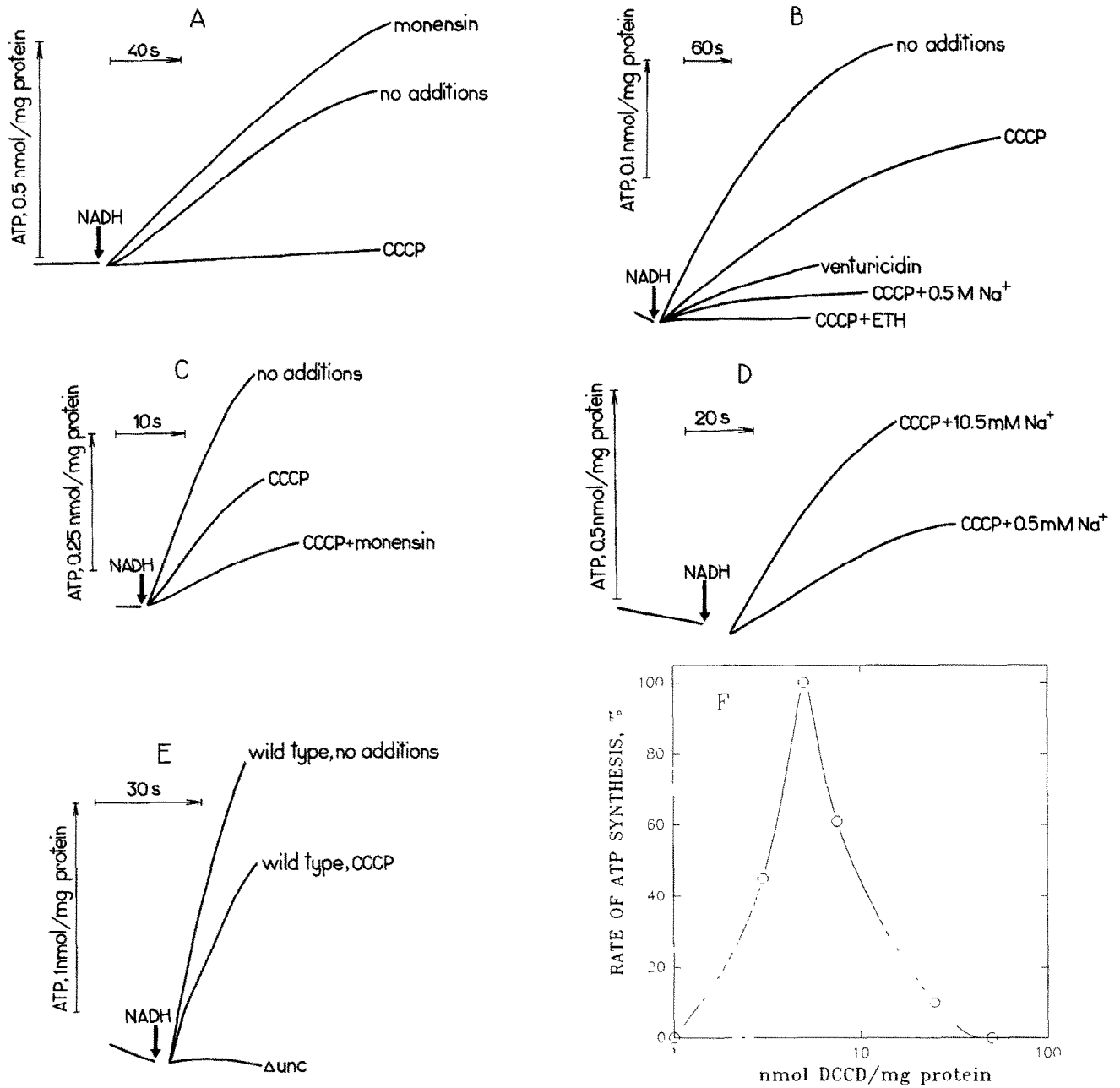

Fig. 2. The ATP synthesis by inverted subcellular vesicles of *E. coli* K-12 GR 70N (A–D, F) or K-12 LE 392 Δunc (E) grown at pH 7.2 (A), at pH 8.6 (B,D,F), at pH 7.2 with 1×10^{-4} M PCP (C) or at pH 8.6 with 1×10^{-4} M PCP and 0.4% glycerol (E). The reaction was started by adding 5 mM NADH. Where indicated, the mixture was supplemented with 1×10^{-5} M CCCP, venturicidin (10 μg/ml protein), 1×10^{-4} M ETH 157, 1×10^{-4} M monensin or Na_2SO_4 (concentration of Na^+ is indicated). In (A–E), the vesicles were pre-treated with DCCD as described in section 2. In (B), the incubation mixture was supplemented with 10 mM AMP.

E. coli was grown at low $\Delta\bar{\mu}_H{}^+$, i.e. in the presence of uncoupler PCP (Fig. 1B) or in alkaline medium (Fig. 1C). Now CCCP was stimulating, rather than inhibiting. The Na^+ uptake was inhibited by the artificial Na^+/H^+ antiporter monensin (Fig. 1B). The Na^+ uniporter ETH 157 (Fig. 1C), the F_0 inhibitor venturicidin (Fig. 1C) and the F_1 inhibitor aurovertin (not shown) were found to suppress the CCCP-stimulated Na^+ uptake.

These observations could be accounted for by suggesting that, at low $\Delta\bar{\mu}_H{}^+$, an ATPase becomes competent in Na^+ pumping. The Na^+-ATPase-generated $\Delta\Psi$ is discharged by CCCP and this allows large-scale Na^+ uptake to occur (for discussion, see [2]).

For the ATP-driven Na^+ transport, low and high concentrations of DCCD proved to be stimulatory and inhibitory, respectively (not shown). This was most probably due to the fact that the vesicles were partially deprived of factor F_1 and, hence, leaky [2].

NADH (but not ATP)-dependent Na^+ uptake was found in the vesicles from the Δunc strain grown with PCP at alkaline pH (Fig. 1D).

In Fig. 2 results of oxidative phosphorylation measurements are shown. If the vesicles were obtained from *E. coli* grown at high $\Delta\bar{\mu}_{H}{}^+$ (neutral pH, no uncoupler), the oxidative phosphorylation was completely inhibited by CCCP whereas monensin was slightly stimulatory (Fig. 2A). If *E. coli* was grown at high pH (Fig. 2B) or with PCP (Fig. 2C), a CCCP-resistant portion of the ATP synthesis appeared. In fact, in the latter case, CCCP decreased the rate of oxidative phosphorylation but still this rate was quite measurable (Fig. 2B–E). The CCCP-resistant phosphorylation proved to be sensitive to monensin, ETH 157, venturicidin (Fig. 2B,C) and aurovertin (not shown). For aurovertin, $C_{1/2}$ was equal to 5 μg/mg protein. The effect of DCCD on ATP formation was biphasic (Fig. 2F), like that on the Na^+ transport. All the data shown in Fig. 2 were obtained with the luciferin-luciferase method. Measurements of the $^{32}P_i$ incorporation into glucose-6-phosphate in the presence of glucose and hexokinase gave similar results (not shown).

To explain these data, one may assume that the Na^+-transporting F_0F_1-type ATPase is competent in ATP synthesis when $\Delta\bar{\mu}_{Na}{}^+$ is formed by the Na^+-motive respiratory chain induced at low $\Delta\bar{\mu}_{H}{}^+$ [2,3]. It is not surprising that CCCP, which is stimulatory for the ATPase-driven Na^+ uptake, partially inhibits the oxidative ATP synthesis in the same vesicles. In the latter case, there is no need to convert $\Delta\Psi$ to ΔpNa. Even more, such conversion may result in some decrease of the total $\Delta\bar{\mu}_{Na}{}^+$ (and, hence, of the ATP synthesis) due to Na^+ leakage when high ΔpNa is formed. To minimize such unfavorable effect of CCCP, artificial ΔpNa (high $[Na^+]_{in}$)andΔpH(low$[H^+]_{in}$)wereimposedonthemembrane of the vesicles in the experiments shown in Fig. 2A–C,E,F. In agreement with the above reasoning, the ΔpNa decrease by adding 0.5 M Na^+ was found to be inhibitory for the CCCP-resistant ATP synthesis (Fig. 2B). On the other hand, addition of 10 mM Na^+ proved to be stimulatory for vesicles which were not loaded with Na^+ (Fig. 2D).

The inhibitor pattern of the $\Delta\bar{\mu}_{Na}{}^+$-dependent ATP synthesis, i.e. its sensitivity to DCCD, venturicidin and aurovertin, indicates that this process is catalyzed by an F_0F_1-type enzyme which is similar (or even identical) to that carrying out the $\Delta\bar{\mu}_{H}{}^+$-dependent oxidative phosphorylation in *E. coli* grown at high $\Delta\bar{\mu}_{H}{}^+$. Such an assumption was supported by finding that the *E. coli* Δunc B-D mutant lacking F_0F_1 ATP-synthase fails to catalyze the ATP-dependent Na^+ uptake (Fig. 1D) and the $\Delta\bar{\mu}_{Na}{}^+$-dependent ATP formation (Fig. 2E). In these experiments, the mutant cells were grown on the alkaline medium (see section 2) supplemented with 0.4% glycerol and PCP.

Thus one may conclude that growth at high pH or in the presence of uncoupler results in a modification of the F_0F_1-type H^+-ATP-synthase such that it becomes competent in the Na^+-driven ATP synthesis.

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